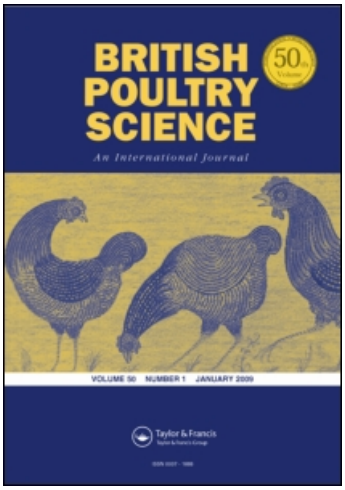

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In vitro effects of plant and mushroom extracts on immunological function of chicken lymphocytes and macrophages

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In vitro effects of plant and mushroom extracts on immunological function of chicken lymphocytes and macrophages[1]

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Abstract 1. The present study was conducted to examine the effects of organic extracts from milk thistle (*Silybum marianum*), turmeric (*Curcuma longa*), reishi mushroom (*Ganoderma lucidum*), and shiitake mushroom (*Lentinus edodes*) on innate immunity and tumor cell viability.

2. Innate immunity was measured by lymphocyte proliferation and nitric oxide production by macrophages, and the inhibitory effect on tumor cell growth was assessed using a non-radioactive assay. For measuring the cytokine levels in the HD11 macrophages which were treated with extracts of turmeric or shiitake mushroom, the levels of mRNAs for interferon-α (IFN- α), interleukin-1β (IL-1β), IL-6, IL-12, IL-15, IL-18, and tumor necrosis factor superfamily 15 (TNFSF15) were quantified by real time RT-PCR.

3. *In vitro* culture of chicken spleen lymphocytes with extracts of milk thistle, turmeric, and shiitake and reishi mushrooms induced significantly higher cell proliferation compared with the untreated control cells. Stimulation of macrophages with extracts of milk thistle and shiitake and reishi mushrooms, but not turmeric, resulted in robust nitric oxide production to levels that were similar with those induced by recombinant chicken interferon-γ. All extracts uniformly inhibited the growth of chicken tumor cells *in vitro* at the concentration of 6·3 through 100 μg/ml. Finally, the levels of mRNAs encoding IL-1β, IL-6, IL-12, IL-18, and TNFSF15 were enhanced in macrophages that were treated with extracts of turmeric or shiitake mushroom compared with the untreated control.

4. These results document the immunologically-based enhancement of innate immunity in chickens by extracts of plants and mushrooms with known medicinal properties *in vitro*. *In vivo* studies are being planned to delineate the cellular and molecular mechanisms responsible for their mechanism of action.

INTRODUCTION

Chicken meat is a major protein source for human nutrition. Over the past several decades, widespread use of antibiotic-based growth promoters has dramatically improved the yield and efficiency of worldwide poultry production. However, due to increasing concerns with prophylactic drug use and its deleterious side effects, much interest has been devoted toward the development of drug-independent control strategies against poultry diseases (Lillehoj and Lee, 2007*a*,*b*). Dietary feeding of plant-derived phytonutrients to enhance protective immunity and increase resistance against poultry diseases, particularly enteric diseases such as coccidiosis, offers a low-cost alternative control method using natural food products (Banfield *et al*., 2002;

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Lee *et al*., 2007*a*, 2008*b*; Naidoo *et al*., 2008). The effects of plant extracts on host defense against microbial infections and tumors have been positively correlated with their ability to enhance various *in vitro* parameters of protective immunity (Park *et al*., 2004; Lee *et al*., 2005, 2007*b*, 2009*a*).

A variety of plant extracts have traditionally been used to enhance resistance to diseases. For example, milk thistle (*Silybum marianum*) has been used for centuries as a natural remedy for hepatic and biliary tract diseases (Valenzuela *et al*., 1985; Flora *et al*., 1998; Rambaldi *et al*., 2005) and is one of the most commonly used herbs in North America (White *et al*., 2007). Silymarin, derived from the milk thistle, and its main active constituent, silybin, decreased the activity of tumor promoters (Agrawal *et al*., 1994), acted as anti-oxidants to scavenge free radicals, and inhibited lipid peroxidation (Bosisio *et al*., 1992; Carini *et al*., 1992; Mira *et al*., 1994). El-Kamary *et al*. (2009) reported that while standard recommended doses of silymarin were safe, its immunomodulatory effects at these concentrations were negligible. Plants of the genus *Curcuma*, including *C. longa* (turmeric), showed anti-oxidative and anti-inflammatory properties, and compounds isolated from *C. amada* and *C. caesi* were shown to inhibit the growth of Gram positive and negative bacteria (Policegoudra *et al*., 2007; Sodsai *et al*., 2007; Mannangatti and Narayanasamy, 2008). *Lentinus edodes* (shiitake mushroom) has been studied for its medicinal benefits, particularly its anti-tumor and anti-viral properties and as possible treatments for atopic diseases and arthritis (Surh and Lee, 1996; Gbolade *et al*., 1997; Park *et al*., 2004). Reishi mushroom (*Ganoderma lucidum*) enhanced the protective immune response of carp against *Aeromonas* bacteria (Yin *et al*., 2009). Extracts of reishi up-regulated cell-mediated immunity and pro-inflammatory cytokine production, and alleviated the effects of cyclophosphamide administration on body weight loss, natural killer and cytotoxic T cell activities, and interferon (IFN)-γ production (Yin *et al*., 2009). Given the prior studies that demonstrated the immunomodulatory properties of milk thistle, turmeric, shiitake, and reishi in humans and various animal models, the current investigation was conducted to examine their effects on innate immunity and tumor cell viability in chickens.

MATERIALS AND METHODS

Preparation of samples

The extracts of turmeric, milk thistle, shiitake, and reishi were obtained from Pancosma S.A. (Geneva, Switzerland). Milk thistle and turmeric were extracted with organic solvents and spray-dried. Aqueous extract of shiitake and reishi mushrooms were prepared at 95°C with pressure and spray-dried. All dried samples were dissolved in PBS, pH 7·2 as 200 μg/ml stock solutions, sterilized by membrane filtration (0·45 μm), and stored at −80°C until use. Working dilutions were prepared in PBS.

Spleen lymphocyte proliferation

All experiments were performed according to the guidelines established by the Beltsville Area Animal Care and Use Committee. Spleens of 3-week-old specific pathogen-free Ross/Ross broilers (Longenecker's Hatchery, Elizabethtown, PA) were removed and placed in Petri dishes with 10 ml of Hanks' balanced salt solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Single cell preparation and lymphocyte proliferation were carried out as described (Lee *et al*., 2007*a*, 2008*b*). In brief, splenic lymphocytes were prepared by gently flushing through a cell strainer and single cells were purified using Histopaque-1077 (Sigma) density gradient medium by centrifugation. Isolated splenocytes were adjusted to 1×10^7 cells/ml in RPMI-1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and 100 μl/well were added to 96-well flat bottom plates with 100 μl/well of milk thistle, turmeric, shiitake, or reishi extracts (16, 8, 4, 2, and 1 μg/ml), 0·5 μg/ml of concanavalin A (Con A, Sigma) as a positive control, or medium alone as a negative control. The cells were incubated at 41°C in a humidified incubator (Forma, Marietta, OH) with 5% CO_2 for 48 h and cell numbers were measured using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] as described by the manufacturer (Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) by measuring optical densities at 450 nm using a microplate spectrophotometer (BioRad, Hercules, CA).

Tumor cell cytotoxicity

RP9 chicken tumor cells, a retrovirus-transformed B cell line (Hong *et al*., 2006*a*; Lee *et al*., 2009*a*), were cultured at 1×10^6 cells/ml (100 μl/well) in 96-well plates with 100 μl/well of milk thistle, turmeric, shiitake, or reishi extracts (100, 50, 25, 12·5, and 6·3 μg/ml), recombinant chicken NK-lysin (1·0 μg/ml) (Hong *et al*., 2006*a*) as a positive control, or medium alone as a negative control at 41°C in a humidified incubator supplemented with 5% CO_2 for 48 h.

Table. *Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines*

RNA target	Primer sequences		PCR product size (bp)	Genebank accession no.
	Forward	Reverse		
GAPDH	5′-GGTGGTGCTAAGCGTGTTAT-3′	5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
IFN-α	5′-GACATCCTTCAGCATCTCTTCA-3′	5′-AGGCGCTGTAATCGTTGTCT-3′	238	AB021154
IL-1β	5′-TGGGCATCAAGGGCTACA-3′	5′-TCGGGTTGGTTGGTGATG-3′	244	Y15006
IL-6	5′-CAAGGTGACGGAGGAGGAC-3′	5′-TGGCGAGGAGGGATTTCT-3′	254	AJ309540
IL-12	5′-AGACTCCAATGGGCAAATGA-3′	5′-CTCTTCGGCAAATGGACAGT-3′	274	NM_213571
IL-15	5′-TCTGTTCTTCTGTTCTGAGTGATG-3′	5′-AGTGATTTGCTTCTGTCTTTGGTA-3′	243	AF139097
IL-18	5′-GGAATGCGATGCCTTTTG-3′	5′-ATTTTCCCATGCTCTTTCTCA-3′	264	AJ277865
TNFSF15	5′-CCTGAGTATTCCAGCAACGCA-3′	5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	AB194710

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; TNFSF15, tumour necrosis factor superfamily 15.

Following incubation, cell numbers were measured using WST-8.

Nitric oxide (NO) production by macrophages

HD11 macrophages were cultured at 1×10^5 cells/ml (100 µl/well) in 96-well plates with 100 µl/well of milk thistle, turmeric, shiitake, or reishi extracts (100, 50, 25, 12·5, and 6·3 µg/ml), recombinant interferon-γ (1·0 µg/ml) as a positive control (Lillehoj and Choi, 1998; Lee *et al*., 2008*a*, 2009*a*,*c*), or medium alone as a negative control in a humidified incubator at 41°C and 5% CO_2 for 24 h. Following incubation, 100 µl of cell culture supernatants were transferred to fresh flat-bottom 96-well plates, mixed with 100 µl of Griess reagent (Sigma), and the plates were incubated for 15 min at room temperature. The optical densities were measured at 540 nm using a microplate reader and nitrite concentrations (µM) were determined using a standard curve generated with known concentrations of sodium nitrite.

Quantification of cytokine mRNA levels

HD11 macrophages were treated with extracts of turmeric or shiitake mushroom (100 µg/ml) in a humidified incubator at 41°C and 5% CO_2 for 24 h and the levels of mRNAs for interferon-α (IFN-α), interleukin-1β (IL-1β), IL-6, IL-12, IL-15, IL-18, and tumor necrosis factor superfamily 15 (TNFSF15) were quantified by real time RT-PCR as described (Hong *et al*., 2006*b*,*c*; Lee *et al*., 2008*b*, 2009*b*). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), and 5·0 µg of total RNA were treated with 1·0 U of DNase I (Sigma), the mixture was incubated for 15 min at room temperature, 1·0 µl of stop solution was added to inactivate DNase I, and the mixture was heated at 70°C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, 5·0 µg of RNA was combined with 10X first strand buffer, 1·0 µl of oligo (dT) primer (5·0 µg/µl), 0·8 µl of dNTP mix (25 mM of each dNTP), and RNase-free water was added to a total volume of 19 µl. The mixture was incubated at 65°C for 5 min, cooled to room temperature, 50 U of StrataScript reverse transcriptase was added, the mixture was incubated at 42°C for 1 h, and the reaction was stopped by heating at 70°C for 5 min. Quantitative RT-PCR oligonucleotide primers for chicken cytokines and the GAPDH internal control are listed in the Table. Amplification and detection were carried out using equivalent amounts of total RNA with the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA. Levels of individual transcripts were normalized to those of GAPDH by the Q-gene program (Muller *et al*., 2002). To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression prior to calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression (MNE) using the Q-gene program (Lee *et al*., 2008*b*, 2009*b*).

Statistical analyses

Each sample was performed in triplicate. Statistical analyses were performed using SPSS software (SPSS 15·0 for Windows, Chicago, IL), and all data were expressed as mean ± SD or mean ± SEM values. Mean values of negative control-treated and extract-treated samples were compared by one-way analysis of variance (ANOVA) or the *t*-test and differences were considered statistically significant at $P < 0{\cdot}05$.

RESULTS

Spleen lymphocyte proliferation

Milk thistle extract stimulated splenocyte proliferation at 4, 2, and 1 µg/ml compared with the

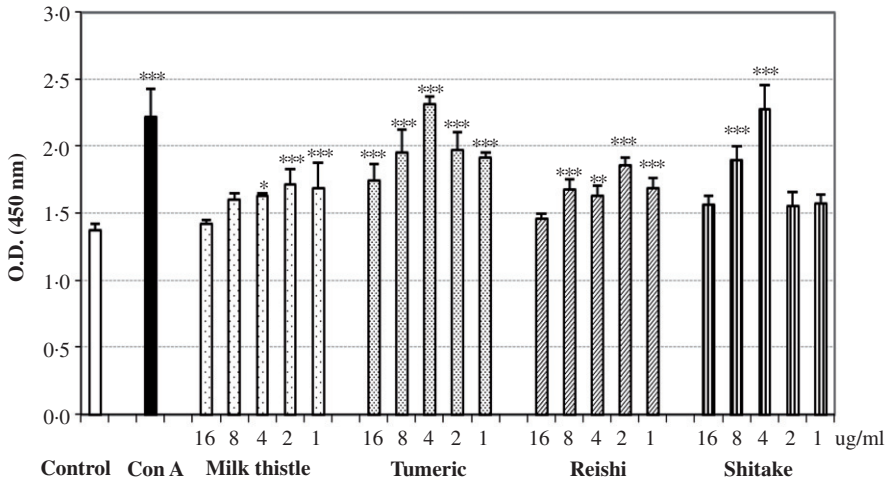

Figure 1. *Effect of milk thistle, turmeric, and shiitake and reishi mushroom extracts on splenocyte proliferation. Spleen cells were cultured with the indicated concentrations of each extract, Con A (0·5 μg/mL), or medium alone as a negative control for 48 h and cell numbers were measured by the WST-8 assay as described in the Materials and Methods section. Each bar represents the mean ± SD value obtained from triplicate culture wells. Each value was compared by t test with the medium alone control. Significant differences are indicated as *P < 0·05, **P < 0.01, and ***P < 0·001.*

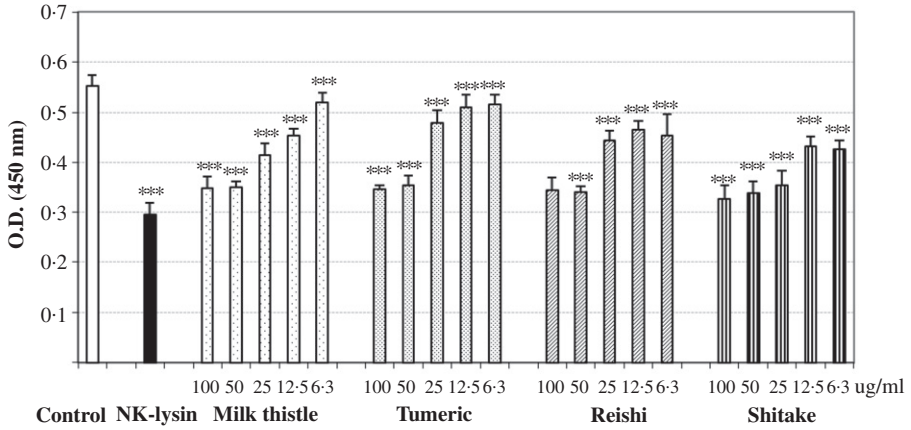

Figure 2. *Effect of milk thistle, turmeric, and shiitake and reishi mushroom extracts on tumour cell viability. RP9 tumour cells were cultured with the indicated concentrations of each extract, NK-lysin (1·0 μg/ml), or medium alone as a negative control for 48 h and cell numbers were measured by the WST-8 assay as described in the Materials and Methods section. Each bar represents the mean ± SD value obtained from triplicate culture wells. Each value was compared by t test with the medium alone control. Significant differences are indicated as ***P < 0·001.*

medium control and turmeric extract increased proliferation at all of the concentrations tested (Figure 1). Extracts of reishi and shiitake mushrooms increased splenocyte proliferation at 8 and 4 μg/ml. Turmeric extract showed the greatest stimulatory activity, being similar to that induced by 0·5 μg/ml of Con A. No toxic effects of the plant or mushroom extracts on spleen cells were observed at any of the concentrations tested.

Tumor cell cytotoxicity

All extracts decreased RP9 tumor cell viability compared with the media controls (Figure 2). The cytotoxic effects were dose-dependent and at 100 or 50 μg/ml were similar to that of the NK-lysin positive control.

NO production by macrophages

Extracts of milk thistle, reishi mushroom, and shiitake mushroom stimulated NO production by HD11 macrophages in a dose-dependent manner compared with the media controls (Figure 3). Milk thistle and reishi mushroom at 100 μg/ml showed more than 70% of NO production compared with the positive control, recombinant chicken IFN-γ.

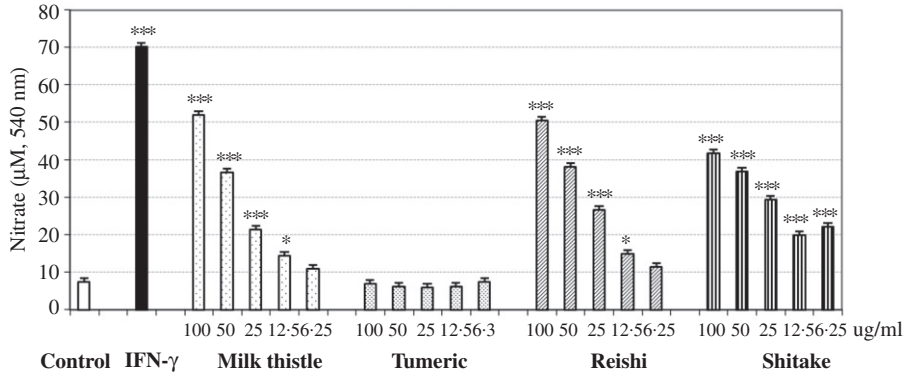

Figure 3. *Effect of milk thistle, turmeric, and shiitake and reishi mushroom extracts on NO production. HD11 macrophages were cultured with the indicated concentrations of each extract, IFN-γ (1·0 μg/ml), or medium alone as a negative control for 24 h and NO in culture supernatants were measured as described in the Materials and Methods section. Each bar represents the mean ± SD value obtained from triplicate culture wells. Each value was compared by* t *test with the medium alone control. Significant differences are indicated as *P< 0·05 and ***P< 0·001.*

Cytokine production

The levels of mRNAs for the pro-inflammatory cytokines IL-1β and IL-6 were higher following treatment with 100 μg/ml of shiitake extract compared with media controls (Figure 4). Transcripts for TNFSF15 were also up-regulated in the cells treated with shiitake mushroom, while significantly higher levels of transcripts for IL-12 and IL-18 were found in the turmeric-treated cells.

DISCUSSION

This study demonstrated that extracts of milk thistle, turmeric, shiitake mushroom, and reishi mushroom activated parameters of innate immunity and inhibited the growth of tumor cells. Milk thistle, turmeric, reishi, and shiitake extracts significantly increased the proliferation of spleen lymphocytes. All extracts also reduced tumor cell viability, and all except tumeric induced NO production by chicken macrophages. A more variable response was seen with the stimulation of inflammatory cytokine production, with shiitake extract increasing the levels of IL-1β, IL-6, and TNFSF15, tumeric increasing the levels of IL-12 and IL-18, and neither extract influencing the levels of IFN-α or IL-15.

T and B lymphocytes, macrophages, monocytes, and NK cells participate in innate and acquired immune defenses. Previous studies have demonstrated that the effects of natural food and herbal products on host defense against microbial pathogens and tumors were directly correlated with their ability to stimulate lymphocyte proliferation (Lee *et al*., 2009*a*,*b*,*c*; Park *et al*., 2004). Splenocyte proliferation stimulated by medicinal fruits and vegetables was attributed to their high concentration of phenolic compounds (Yu *et al*., 2009*a*). Curcumin is a phenolic natural product isolated from the rhizome of *Curcuma longa* (turmeric). Turmeric promotes cell membrane integrity, decreases the expression of pro-apoptotic signaling molecules, and modulates cellular levels of stress-related proteins (Dutta *et al*., 2009). Tumeric extract also inhibited hepatitis B virus replication in hepatocytes without cytotoxic effects (Kim *et al*., 2009). Silibinin is the major active constituent of silymarin, a mixture of polyphenolic flavinoids extracted from milk thistle that was effective in improving the symptoms of acute hepatitis (El-Kamary *et al*., 2009). Silymarin is currently being used clinically in Europe and Asia for the treatment of liver diseases. Shiitake mushroom-derived immunostimulatory lentinan protected against murine malaria blood-stage infection by provoking adaptive immune responses (Zhou *et al*., 2009) and decreased the expression of genes involved in acute inflammatory reactions to inflammatory agents (Djordjevic *et al*., 2009). Extracts of reishi mushroom stimulated leukocyte respiratory burst activity, increased their phagocytic activity, and augmented their production of lysozyme, an anti-microbial enzyme (Yin *et al*., 2009). Feed supplemented with reishi was reported to produce the best growth performance and the highest pseudorabies antibody titers in weanling piglets (Chen *et al*., 2008).

We also observed that milk thistle, turmeric, reishi, and shiitake extracts inhibited the viability of a chicken tumor cell line in a dose-dependent manner, with that shiitake extract showing the greatest effect. While these results suggest a direct cytotoxic effect of the extracts on tumor cells themselves, they do not exclude an additional indirect effect, for example through the potentiation of anti-tumor inflammatory cells. In support of the latter possibility, a xenograft mouse model system was used to show that curcumin inhibited tumor growth *in vivo*

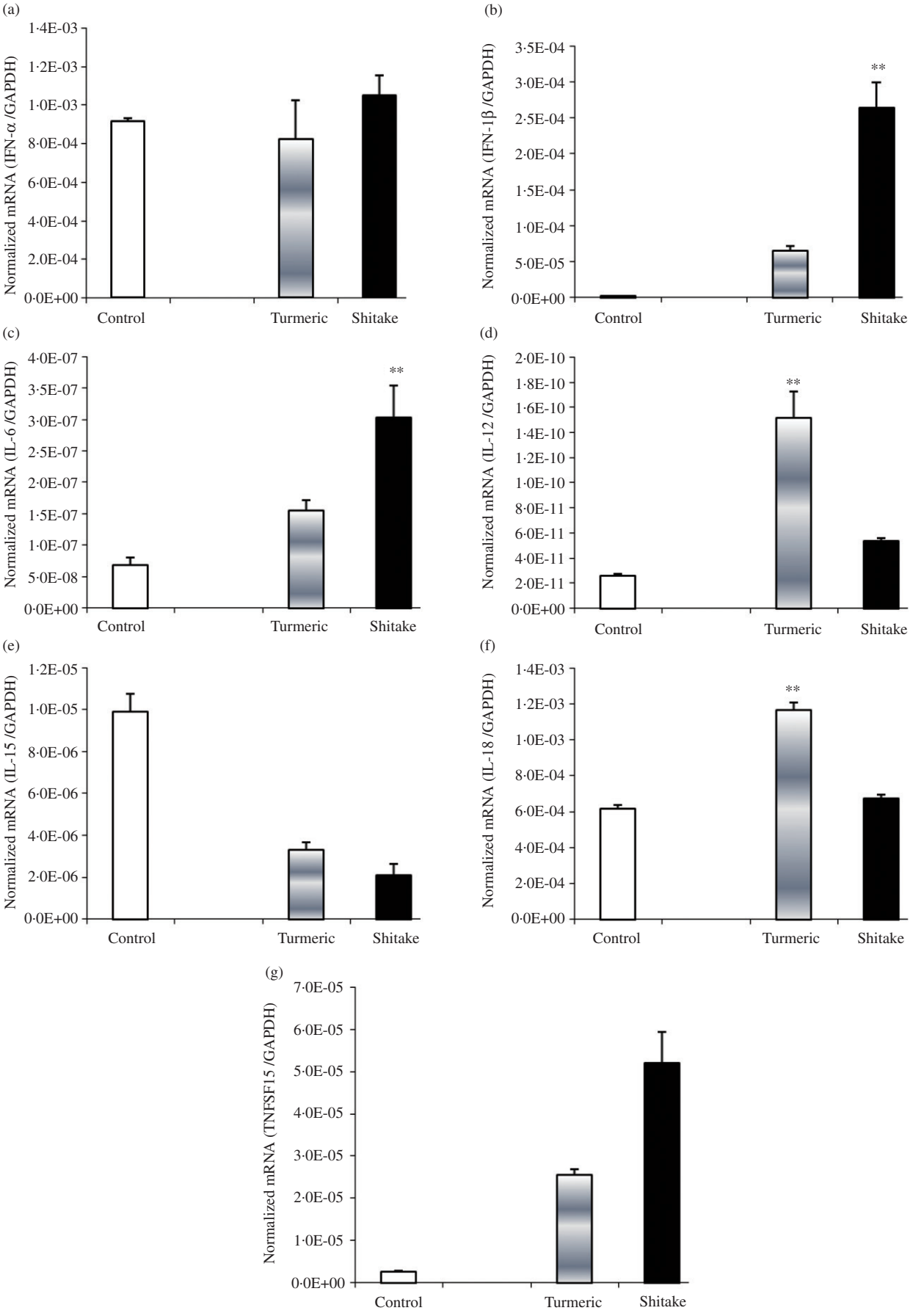

Figure 4. *Effect of turmeric and shiitake mushroom extracts on cytokine mRNA. HD11 macrophages were cultured with 100 μg/ml of each extract or medium alone as a negative control for 24 h and cytokine mRNA was determined by quantitative RT-PCR and normalised to GAPDH mRNA values. Each bar represents the mean ± SEM value. Each value was compared with the medium alone control according to the ANOVA. Significant differences are indicated as **P < 0·01.*

(Li *et al*., 2009). Silibinin was shown to inhibit tumor cell growth and blocked the expression of survivin, a negative regulator of apoptosis, in a laryngeal squamous cell carcinoma cell line (Bang *et al*., 2008). Silymarin showed anti- and pro-mutagenic effects in the Ames bacterial reverse mutation assay, indicating that its effects on eukaryotic gene expression may be mediated through transcriptional regulation (Kaleeswaran *et al*., 2009).

Macrophages play a significant protective role in host defense against infectious agents and tumors, in part, through the elaboration of effecter molecules such as NO (Santoni *et al*., 1999). IFN-γ-stimulated NO production by chicken macrophages has been reported (Okamura *et al*., 2005). Extracts of milk thistle, reishi, and shiitake extracts, but not turmeric, significantly stimulated NO production by HD11 macrophages in a dose-dependent manner that was comparable to that of the IFN-γ positive control. β-Glucan, the major medicinal component of mushrooms, up-regulated the phenotypic functions of macrophages such as phagocytic uptake, production of NO and other reactive oxygen species, cytokine gene expression, and morphological changes (Lee *et al*., 2008*a*, 2009*c*, *d*). Our results showing the stimulatory effect of plant and mushroom extracts on macrophage NO production may be related to the result in our previous *in vivo* study where these extracts increased IFN-γ expression in chickens (Lee *et al*., 2009*b*). Moreover, our data corroborate several previous reports demonstrating that the bioactive properties of several different medicinal foods and herbs were mediated through macrophage activation (Stimpel *et al*., 1984; Sugawara *et al*., 1984; Sakagami *et al*., 1991; Suzuki *et al*., 1994).

Macrophages, T lymphocytes, and other leukocytes produce immunoregulatory cytokines and chemokines that initiate and amplify protective immune responses (Lillehoj and Trout, 1996; Lillehoj, 1998). Cytokines are regulators of host responses to infection, inflammation, and trauma. For example, IL-1β is a major proinflammatory cytokine that is produced by macrophages, monocytes, and dendritic cells, and an important mediator of innate immunity. In mammals, IL-1β increases the expression of adhesion factors on endothelial cells to enable the transmigration of leukocytes from the vasculature to the sites of infection. IL-1β and IL-18 are structurally homologous proteins that play critical roles in initiating inflammation. IL-6 also is produced by T lymphocytes and macrophages and acts as both a pro-inflammatory and anti-inflammatory cytokine (Waldmann and Tagaya, 1999). IL-15 is primarily secreted by mononuclear phagocytes and enhances the activation of memory T cells (Kanegane and Tosato, 1996). We observed higher levels of mRNAs encoding IL-1β, IL-6, IL-12, IL-18, and TNFSF15 in macrophages treated with extracts of turmeric or shiitake. In a previous study using mouse macrophages, shiitake extracts stimulated TNF-α production (Yu *et al*., 2009*b*), and shiitake lentinan increased the production of IL-12 in murine spleen dendritic cells co-cultured with parasitized red blood cells (Zhou *et al*., 2009). Thus, the increased levels of transcripts for several chicken cytokines that we observed suggest that extracts of the plants and mushrooms studied in this report, and their purified bioactive constituents, may enhance protective immunity against poultry infections (Hong *et al*., 2006*c*; Lee *et al*., 2009*d*). Studies examining the potential of these extracts as vaccine adjuvants are currently ongoing in our laboratory.

In conclusion, the current results document the immunologically-based enhancement of innate immunity by extracts of plants (milk thistle, turmeric) and mushrooms (reishi, shiitake) in chickens. Future *in vitro* and *in vivo* studies will be necessary to delineate the cellular and molecular mechanisms responsible for their mechanism of action.

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